TOWARD ELECTROPHORETIC SEPARATION of MEMBRANE PROTEINS in SUPPORTED n-BILAYERS

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TOWARD ELECTROPHORETIC SEPARATION of MEMBRANE PROTEINS in SUPPORTED *n*-BILAYERS

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Abbreviations:

DMPC: 1,2-dimyristoyl-sn-glycero-3-phosphocholine

EggPC: L-α-phosphatidylcholine (Egg, Chicken)

SLB: Supported Lipidic Bilayer

SQR: Sulfide Quinone Reductase

α-HL: Alpha Hemolysin

Keywords:

Electrophoresis, Membrane, Mobility, Proteins, Separation.

Abstract

Membrane proteins are key constituents of the proteome of cells but are poorly characterized, mainly because they are difficult to solubilize. Proteome analysis involves separating proteins as a preliminary step towards their characterization. Currently, the most common method is "solubilizing" them with sophisticated detergent and lipid mixtures for later separation *via*, for instance, sodium dodecyl sulfate polyacrylamide gel electrophoresis. However, this later step induces loss of 3D structure (denaturation).

Migration in a medium that mimics the cell membrane should therefore be more appropriate. Here, we present a successful electrophoretic separation of a mixture first of two and then of three different membrane objects in supported *n*-bilayers. These "objects" are composed of membrane proteins Sulfide Quinone Reductase and α-Hemolysin, model proteins easily handled in aqueous solution before insertion. Sulfide Quinone Reductase forms an object from three monomers together with some detergent and self-inserts into the upper leaflet. α-Hemolysin inserts as a spanning heptamer into a bilayer or can build stable dimers of α-Hemolysin heptamers under certain conditions. By appropriately adjusting pH it proved possible to move them in different ways. This work holds promise for separating membrane proteins without losing their 3D structure, within a lipidic environment that is more physiologically realistic.

1 Introduction

Membrane proteins play an important role in biological processes. Located at the boundary surface between the inside and the outside of the cell, they play many roles, acting as a gate in active/passive transport of ions/molecules through membranes and thereby ensuring reception/transmission of signals, modeling and adhesion, intercellular recognition, extracellular matrix binding, enzymatic activity, etc. Although they constitute roughly 30% of the proteome of a mammalian cell, i.e. close to 30 000 proteins, only 999 (≈ 3.3 %) 3D structures are known [1]. Yet membrane proteins represent 50% of pharmaceutical targets, knowledge of 3D structure being crucial to researchers seeking to design better-targeted drugs. This lack of characterization stems from issues with purifying, crystallizing and functionally reconstituting membrane proteins. The main reason is that they are extremely difficult to handle in a water environment without denaturing them. In practice, a mixture of detergents and lipids must be used to stabilize the 3D structure. In addition, the environment is very difficult to reproduce in a fully biomimetic manner, and can wind up being completely different, for example with a separation method like SDS-PAGE. In that case, because of denaturing conditions, the 3D structure is completely lost. As a successful alternative, methods like Blue Native and Clear Native PAGE [2-6] involve one-step isolation of membrane proteins using nonionic detergents such as dodecyl maltoside, Triton X-100 or digitonin under conditions enabling biological membranes to solubilize biological membranes. By carefully selecting and adjusting the detergent and its concentration, it is possible to create conditions that are not harmful to the membrane proteins or the complexes, owing to the presence of some of the lipids from the solubilized membranes. Then, separated bands can be excised and run in another dimension for final separation with various denaturing techniques (SDS-PAGE, doubled SDS-PAGE and IEF/SDS 3D PAGE.

Some years ago another strategy was used in [7]. In this work, the authors were able to report progress in the knowledge of membrane proteins, by identifying complexes playing vital biological roles in *Saccharomyces cerevisiae*. They identified complexes with 1590 putative integral, extrinsic and lipid-anchored membrane proteins using tandem affinity purification and mass spectrometry. However, most of the work clearly remains to be done.

More recently Okamoto et al. separated for the first time two membrane pore-forming proteins in multilayer lipid membranes at one pH [8]. Embedding the membrane proteins in a medium which partially mimics their natural environment meant that they could be separated without denaturation.

Bao et al have reported a 25-fold increase concentration of trans-membrane protein–proteorhodopsin incorporated into supported lipid bilayers using electric fields [9]

We propose here to carry out electrophoretic separation of a mixture of two different membrane proteins at differing pHs in differing lipidic systems (different chemical nature and number of bilayers) and to separate three objects: the two previous proteins plus a dimer of one of them. We use supported phospholipidic *n*-bilayers of two different lipids with *n* which can be set to 1, 2 or ≅ 35. One single bilayer (n=1) constitutes the basic system. A double bilayer (n=2) provides another test system. A multibilayer (n ≅ 35), which has the advantage of being very easy to build, provides a third test system and may improve membrane protein loading capacities for what we hope will become routine separation.

In previous papers [10-13] we measured some physico-chemical properties (diffusion coefficient, electrophoretic mobility, *pI* and charge) of two different membrane proteins, α-Hemolysin (α-HL) and Sulfide Quinone Reductase (SQR), taken as models in such an environment. They were chosen because they are very different: one is monotopic (inserts in one leaflet); the other is transmembranar (spans the two leaflets).

This study, using an easy-to-build system, confirms this method as one that can be routinely used to carry out real electrophoretic separation of membrane proteins, starting from a cell, a cell lysate, or other sources. It illustrates the predictive nature and the versatility of the system, based on the intrinsic properties of each protein. Starting with a mixture of known objects, conditions can be chosen so as to immobilize either one or the other object, move them in opposite directions, etc. Later, starting with an unknown mixture, all separating conditions are "open", to be chosen carefully in accordance with each situation, especially the appropriate pH.

2 Materials and Methods

2.1 Sample

2.1.1. Supports

The slides used as supports were 2.5x 2.5 cm², either mica cleaved on both sides just before use (JBG-Metafix, France) to support the double bilayer or glass (Marienfield, cut edges, France) to support the single bilayer or the multibilayer. They were cleaned by immersion for 10 min in freshly prepared alcoholic NaOH, then thoroughly rinsed with ultra-pure water and sonicated 3 times for 5 min in ultra-pure water. The advantage of this cleaning technique lies in dissolving an ultrathin layer of glass, giving access to a fresh surface, with immediately constant zeta potential.

2.1.2. Lipids

All lipids were purchased from Avanti Polar Lipids (Coger, France) and used without further purification. Supported single and double bilayers were prepared using 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Mw 734 g·mol^{-1}). Supported multibilayers were prepared using either DMPC or L-α-phosphatidylcholine (EggPC, Mw 770 g·mol^{-1}).

The single bilayer and double bilayer of DMPC were built using the Langmuir-Blodgett transfer technique (for details, see [14]). Lipid molecules solubilized in chloroform (~1 mg/ml) were deposited on the water subphase (18 MΩcm, MilliQ, 15 °C) of a Langmuir trough (KSV Minitrough 361 mm × 74 mm, Finland), equilibrated for 10 minutes to allow complete solvent evaporation and then compressed to 30 mN/m.

For the single bilayer on glass, the first monolayer was transferred using the Langmuir-Blodgett technique. The second layer was transferred using the out-of-equilibrium Langmuir-Schaefer (LS) technique: carrying the first monolayer, the "hydrophobic" support was rotated in air from the vertical to the horizontal position, and lowered through the air/water interfacial monolayer of the trough. Transfer ratios were close to 1.

For the double bilayer on mica, the fourth monolayer, closing the second bilayer, was also transferred using the Langmuir-Schaefer (LS) technique: carrying the first three monolayers, the "hydrophobic" support was rotated in air and lowered through the air/water interfacial monolayer of the trough. Transfer ratios were close to 1 for the first three monolayers and 0.8 for the fourth monolayer.

The multibilayer was formed *via* the lipid film hydration method. 20 µL of lipids (either DMPC or EggPC) in chloroform (1mg/mL) were first desiccated under vacuum for 1 h on a glass slide, the whole contained in a desiccator. Then the slide with the film was positioned in our incubation cell (a Teflon™ cylinder embedded in steel, 1 cm diameter) and the films were rehydrated at room temperature with ultrapure water (18 MΩ·cm, MilliQ) for 30 min. Obtaining a multibilayer can be difficult. Several articles detail how Multilamellar Vesicles (MLV) can be produced *via* rehydration of dry lipidic film, which usually involves supplying energy to the system, for instance through strong agitation or electric fields. Failing this, the outermost bilayer of a rehydrated DOPC film will spontaneously peel off (possibly bilayer by bilayer). However, it is also reported that peeling does not occur when starting from DPPC in gel phase [15]. More recently, Garcia-Meynes et al [16] carried out AFM experiments on supported rehydrated multibilayer stacks in buffer solution. They kept the same buffer without any rinsing to avoid detachment of the lipid stacks due to turbulence.

In our system, the following should ensure that we have a supported multibilayer:

- Removal, washing and addition to the Teflon cell were performed very gently and along the wall of the cell, to minimize turbulence.
- Working in pure water on glass helps stabilize the rehydrated multibilayer at the glass surface (low ionic strength and good affinity).
- Qualitatively, under the microscope, the sample appeared flat: there was no need to adjust the *z* direction to remain in focus, and no sign of a slowly "swimming" MLV.

Additionally, the electrophoretic mobilities of our objects were analogous when measured several times in single or double bilayers. Experiments were performed in replicate reproducibly in all systems (at least twice, sometimes more).

Assuming a lipid head molecular area of 0.63 nm² [17], given the amount of dried lipids (20 µL from a solution 1mg/mL) and the area exposed to rehydration (a circle 1 cm in diameter), we estimated from the initial quantity of lipid a number of bilayers close to ≈ 35 in the multibilayer. Supported Lipidic Bilayers (SLB) were kept under water throughout and used directly after preparation.

2.1.3. Proteins

α-Hemolysin **(**α-HL**)**, extracted from *Staphylococcus aureus*, was purchased from Sigma Aldrich Inc and used without further purification.

Sulfide Quinone Reductase (SQR) was extracted from *A. aeolicus* membrane with dodecyl-β-D-Maltoside (DDM) and purified as previously described [18]. Following purification, SQR was dialyzed against 50 mM HEPES buffer pH 7.2, 0.1% DDM using Vivaspin ultrafiltration spin columns (Sartorius stedim), concentrated at about 2 mg/ml and stored at -20 °C.

2.1.3.1 Protein Labeling

We labeled the proteins using a kit from Molecular Probes (Invitrogen) to covalently link Alexafluor™ 488 fluorescent dye to the protein, as described in [19]. The initial concentration of the protein was 0.4 mg/ml in PBS 1x (with DDM in the case of SQR), the reaction time was 15 min for α-HL and 40 min for SQR. This yields an average labeling of 0.2 mole Alexa 488/mole protein.

After purification, the solution containing the labeled protein was mixed with the proper amount of a solution of the same protein unlabeled, to reach a final solution of α-HL with 1/7 labeled monomer and a solution of SQR with 1/3 labeled monomer. This rather low-labeled monomer

content rules out any disturbance of the insertion mechanism of the protein and of the dynamics of the final object. Final aliquots of protein (0.2 µM) in Phosphate Buffer (PB) 20mM and NaCl 100 mM pH 7 were stored at -18 °C before use (with DDM in the case of SQR).

2.1.3.2 Protein Insertion

SQR inserts as a trimeric integral monotopic membrane protein at the end of the process [20-21] and is the first object.

Incubated over a fluid bilayer, α-HL monomers in solution assemble as heptameric pre-pores at the surface of the bilayer, turning into transmembrane (spanning) heptameric pores [22-23]. This is the second object.

Incubating α-HL over a lipid bilayer consisting of a mixture of fluid/gel regions (half transition) promotes the formation of dimers of α-HL heptameric pores [10], the third object of our complex protein mixture.

The composition of the aliquots leads on average to one fluorescent label per object (the heptameric pore for α-HL and the SQR trimer for SQR) and to two fluorescent labels per dimer of heptameric α-HL.

2.1.3.3 Preparing a system containing a mixture of 2 objects: SQR and single heptameric α-HL

The volume over a slide with the bilayer positioned in our incubation cell (a Teflon™ cylinder embedded in steel) was 1 ml. First, 700 µl of water were removed, leaving the bilayer under the remaining 300 µl, and 300 µl of an SQR aliquot were added. This final volume of 600 µl of protein (SQR 0.1 µM in PB 10mM NaCl 100 mM, pH 7.2) was incubated for 12 hours at 33 °C (bilayer in the fluid phase), after which 300 µl of the supernatant were discarded and the excess of SQR was gently removed by adding and pipetting 500 µl of distilled water 10 times.

The sample was returned to the incubation cell and again immersed under 1 ml of distilled water, 700 µl of which were removed again. Then 300 µl of an aliquot of α-HL were added to the incubation cell following the same protocol (12 hours at 33 °C).

2.1.3.4 Preparing a system containing a mixture of 3 objects: SQR, single heptameric α-HL, and dimer of heptameric α-HL

The incubation was carried out over a DMPC double bilayer. The first two steps were as described above for the mixture of two objects. Formation of dimers of heptameric α-HL is a

function of temperature and time. To obtain inserted dimers of heptameric α-HL, the solution was brought back to 22 °C, a temperature where gel and fluid lipid regions coexist and which is thus conducive to the formation of dimers of heptameric α-HL. An extra 12 hours of incubation was carried out, which added to the initial 12 h incubation of α-HL gave a total of 24 hours, found to be optimal to obtain a large number of dimers [10].

The different steps were characterized under microscope as:

- Incubation of SQR (12 h at 33 °C)
- Rinsing to eliminate excess SQR
- Incubation of α-HL (12 h at 33 °C)
- Rinsing to eliminate excess α-HL
- Return to 22 °C and re-incubation of a new solution of α-HL: additional 12h at 22 °C (at 30 °C: fluid phase; at 22 °C: coexistence of gel phase with fluid phase, promoting the formation of dimers of heptameric α-HL**)**
- Final rinsing with water

2.1.4 Systems

The different systems of our study are summarized below:

Mixture	**Conditions**	**DMPC single bilayer**	**DMPC double bilayer**	**DMPC Multibilayer**	**EggPC Multibilayer**
of 2 Objects	pH	6.8; 8.3; 9.2	-	6.8; 8.3; 9.2	6.8; 7.2; 8.3; 9.2
	Incubation T(°C)	33	-	33	33
of 3 Objects	pH	-	9.2	-	-
	Incubation T(°C)	-	33 °C (SQR & α-HL) then 22 °C to obtain α-HL dimers	-	-

Table 1: Systems, pH and incubation temperature in different supported lipidic bilayer systems**.**

The temperature used to measure electrophoretic mobility was 35 °C for all systems. This means that measurements were performed with fluid bilayers. For a given species, for a given pH, all mobilities were identical whatever the system (single bilayer, double bilayer, and

multibilayer, composed of EggPC or DMPC) and also identical to the value measured on the object when alone and not in a mixture.

2.2 Videomicroscopy

2.2.1 Cell

The sample (supported bilayer + inserted proteins) was transferred underwater in a thermostated electrophoretic cell. The cell setup was home-made (figure 1): a parallelepipedic tank (30 mm x 25 mm x 5 mm) prepared using two glass plates, giving an interior liquid volume of 3 ml. Near one plate, a Peltier element and a heat sink were used to either cool down or heat the cell. Water in the cell was replaced by gently rinsing and overflowing several times with the buffer (PB 10 mM, NaCl 100 mM and NaN_3 0.2 mM) at the desired pH. The cell was then positioned under microscope to take pictures or to record videos.

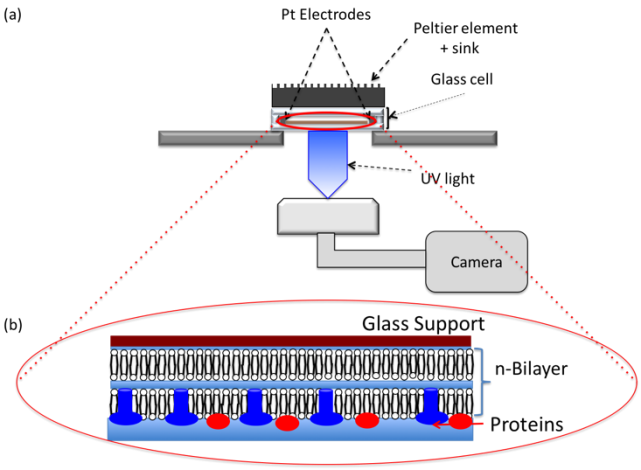

Figure 1: Schematic diagram of the cell used to carry out protein velocity measurements in double SLB: (a) video setup for electrophoretic mobility measurements: cell positioned on an inverted microscope equipped with a camera; (b) zoom on the system: SQR (red ellipses) and α-HL (blue mushrooms) inserted on top of double bilayer.

Platinum electrodes (diameter = 0.25 mm) were positioned in the cell 30 mm apart. The tension was applied between the Pt electrodes, with an electric field of 3 V/cm applied for 20 s. The solvent was replaced after each velocity measurement to remove electrolysis products. This also helped to eliminate any possibility of temperature increase due to Joule heating or pH change; tracking by thermocouple when electric field was on showed no changes.

2.2.2 Video

An inverted fluorescence microscope (Leica) and a camera (Hamamatsu C2400-77: 768 x 494 pixels, 30 MHz) equipped with a fluorescence intensifier suitable for fluorescence imaging

(magnification x 400) were used to carry out protein velocity measurements. As illustrations, series of snapshots extracted from videos at different times (0, 10 and 20 s) are shown in the Results and Discussion section.

Drift velocity measurements were performed as described in Harb et al. [11]. Briefly, the drift velocity of at least 10 spots measured on three different locations from each sample was obtained by dividing distance traveled by the particles by time. Reproducibility was within 10 %. The primary source of error was the measurement of the displacement, due to the resolution of the microscope (± 1µm).

Videos of migration of proteins under electric field (3V/cm) at different pHs are available in Supplementary content file.

2.3 Electrophoretic mobility

The following is a brief reminder of the theoretical basis.

Objects will be separated by electrophoresis when their mobility, namely the ratio of charge q over friction ξ (related to size) are sufficiently different. If, from one object to another, charge and size double conversely, mobility remains unchanged and the objects will not be separated by an electric field. This is what happens with DNA in gel electrophoresis using fields that align DNA molecules (both q and ξ are proportional to DNA length, thus the ratio remains constant [24].

The electrophoretic velocity of charged molecules, $V_{E,}$ is related to the strength of the electric field (E) and electrophoretic mobility (μ_E) as described by eq. #1:

$$V_E = \mu_E E \qquad \text{(eq. \#1)}$$

The resulting effective mobility of the object results from several factors, mainly its intrinsic mobility, the velocity of lipids and a velocity induced by electroosmotic flow (through μ_{EOF}). Thus, the resulting effective mobility of the object (μ_{eff}) should be the sum of all contributions:

$$\mu_{eff} = \mu_E + \mu_{lipid} + \mu_{EOF} \qquad \text{(eq. \#2)}$$

We showed in previous work [9] that μ_{lipid} and μ_{EOF} were negligible in our conditions. We thus get:

$$\mu_{eff} = \mu_E \qquad \text{(eq. \#3)}$$

In other words, we obtain the mobility of the object directly by simply dividing its velocity by the field value.

3. Results and Discussion

3.1 Characterization of the different systems

3.1.1 Mixture of 2 objects

After incubation of SQR, the support was placed under a fluorescence microscope and a density of 426 spots/mm² was observed. After incubation of α-HL and washing, the spot density observed for α-HL was 177 spots/mm².

3.1.2 Mixture of 3 objects

We observed 392/mm^2 spots *versus* 426 spots/mm^2 (mixture of 2) for SQR, a similar reproducibility of insertion to that of the protein alone, which saturated quickly at around 400 spots/mm^2 in [12]. There was the same trend for α-HL with 182 spots/mm^2 *versus* 177 spots/mm^2 (mixture of 2) and ≈180 spots/mm² for the protein alone in [10]. For the dimer of heptameric α-HL, we found 130 spots/mm², which is in good agreement with the 139 spots/mm² obtained after 24 h incubation in [10]. Number of spots varied from one experiment to another by around 5 %.

The mixture of three objects in the bilayer had the following densities: 392/mm^2 of SQR, 182 spots/mm^2 of heptameric α-HL and 130 spots/mm² of dimer of heptameric α-HL. This provides insights into the mechanism of dimer formation: the dimers at a density of 130 spots/mm² self-added to the 182 spots/mm^2 heptameric α-HL already present in the bilayer, forming new objects during this step. Thus, the process of dimer formation appears not to consist in dimerization of already-inserted heptameric α-HL spots.

3.1.3 Identification of objects

Fluorescence intensity in arb.unit.

In previous work [10], we observed an average intensity close to 38 arb.unit. for trimeric SQR and heptameric α-HL. The same intensity was observed here, corresponding to the label content of each object, itself deduced from the labeling procedure. For the dimer of heptameric α-HL alone, the average intensity was ≈ 70 arb.unit., roughly twice the previous intensity, also as expected.

In the mixture of two objects, we observed spots having an average intensity of 38 arb.unit. In the mixture of three objects we observed spots having an average intensity of 38 arb.unit. and spots having an average intensity of 67-70 arb.unit., indicating the presence of the three objects.

Differentiation between SQR and α-HL

For both mixtures (2 or 3 objects):

- SQR was incubated first, then videoed and analyzed. Recording free particle trajectories with our video setup, and treating them with Image J developed by the MOSAIC GROUP at ETH Zurich [25], we determined diffusion coefficients D of SQR at zero electric fields (with the plugin), finding values of D in good agreement with previous reports [12].
- Next, α-HL was incubated. We observed two populations, each with its own mobility, in agreement with those measured on protein alone [11]. This enabled us to distinguish SQR from α-HL.
- No video measurements of diffusion coefficients were performed for α-HL but spot density was observed to have increased, indicating that SQR and α-HL were now inserted in the same bilayer.
- Each determination (fluorescence intensity and electrophoretic mobility) was carried out on 10 spots at least. Using these determinations, we obtained a position pattern for a given object that was then used, in a sort of feedback loop, to selectively color the spots on the figures of the article (SQR in red, α-HL in blue) for easier discrimination.

3.2 Relevant pHs for protein separation

It is important to note again that, although all our objects are membrane objects, they differ significantly in ways that should lead to differing mobilities.

The first object, monotopic, results from the insertion of an assembly of three SQR monomers and is lipid-anchored in only one leaflet. The second object results from the assembly of 7 α-HL monomers initially gathering as a pre-pore at the surface of the bilayer; then this object, turning inside-out like a glove finger, turns into a «mushroom» with its stem completely spanning the two leaflets of the bilayer. This heptamer can dimerize, depending on the inserting condition, and form a stable dimeric complex, the third object of this study.

Our previous studies on trimeric SQR and heptameric α-HL alone provided data on how each protein varies in electrophoretic mobility as a function of pH, enabling us to draw a diagram comparing them (Figure 2). Our aim here was to visualize, and thus to predict, where the pH could be used to separate the objects, *i.e.* by selecting a pH such that one object would go in one direction and the other in the opposite direction, or that one object would be immobile and the other mobile, or that one object would move faster than the other.

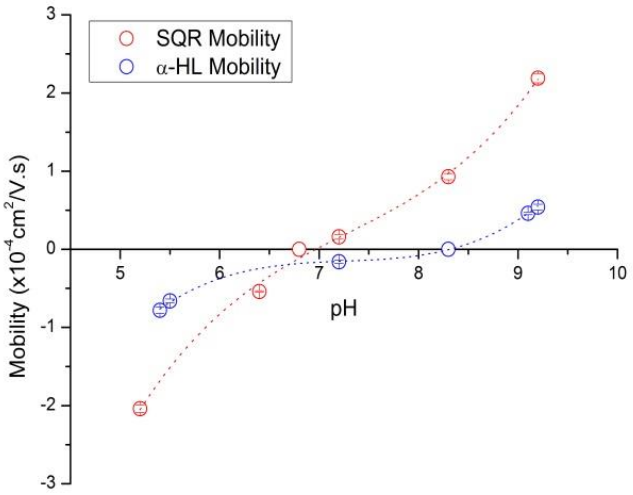

Figure 2: Electrophoretic mobility of trimeric SQR alone and heptameric α-HL alone as a function of pH (the error bar is ±10%).

The results shown in figure 2 enabled us to determine 4 relevant pH values for testing:

- 7.2, where objects should move in opposite directions;

- 9.2, where the two (then the three) objects should race each other in the same direction and should separate, SQR being the fastest.

- 6.8 and 8.3 (SQR and α-HL, respectively), where one object should be immobile because the pH corresponds to its *pI*;

To help visualize the differing behaviors, a series of snapshots is presented in figures 3 to 6. SQR objects are colored red and α-HL objects blue, as are the arrows illustrating their motion.

3.3 Separation of 2 objects (trimeric SQR + heptameric α-HL)

Figure 3 shows a succession of three snapshots taken from the video recording the behavior of the objects at pH 7.2 as a function of time at 0 s, 10 s and 20 s. The vertical red dotted line is an arbitrary reference to highlight the position change of SQR (red spots) and α-HL (blue spots) during the application of the electric field. Length of arrows increases or decreases as the spots become closer to or farther from the vertical dotted line. Ellipses illustrate the motion of one object compared to the other.

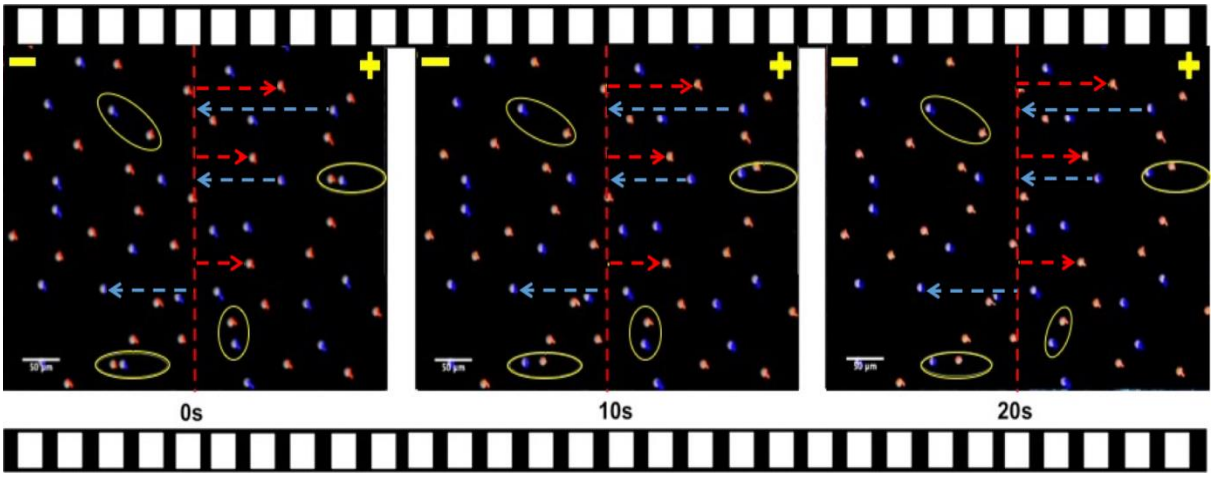

Figure 3: Images of fluorescent SQR and α-HL migration under a 3 V/cm electric field in a buffer solution at pH = 7.2 and recorded at 0, 10 and 20s extracted from video 1. The vertical red dotted line is an arbitrary reference to highlight the position change of SQR and α-HL during the application of the electric field. Ellipses illustrate the relative motion of one object compared to the other. The scale bar is 50 µm.

Figure 3 shows that both proteins have mobility, but in opposite directions since they have opposite charges. The complete sequence is shown in video 1. In our conditions they frequently appear to simply go their own way, ignoring the existence of other species. Moreover, there are some situations where these two different objects approach each other closely enough to collide, with potentially varying results: they could join together and stop migrating or they could migrate as one object with a mobility that is derived from their opposite mobilities.

We were able to capture these particular situations (bottom/left and top/right around t=4s in video 1). We focus on one of these events from the movie, detailed in figure 4: we extracted some snapshots, time interval 2.3 s to 6.8 s, from video 1. In the movie, the objects start to bump into each other at first, bounce, and finally manage to find a way to glide past each other before following separate paths.

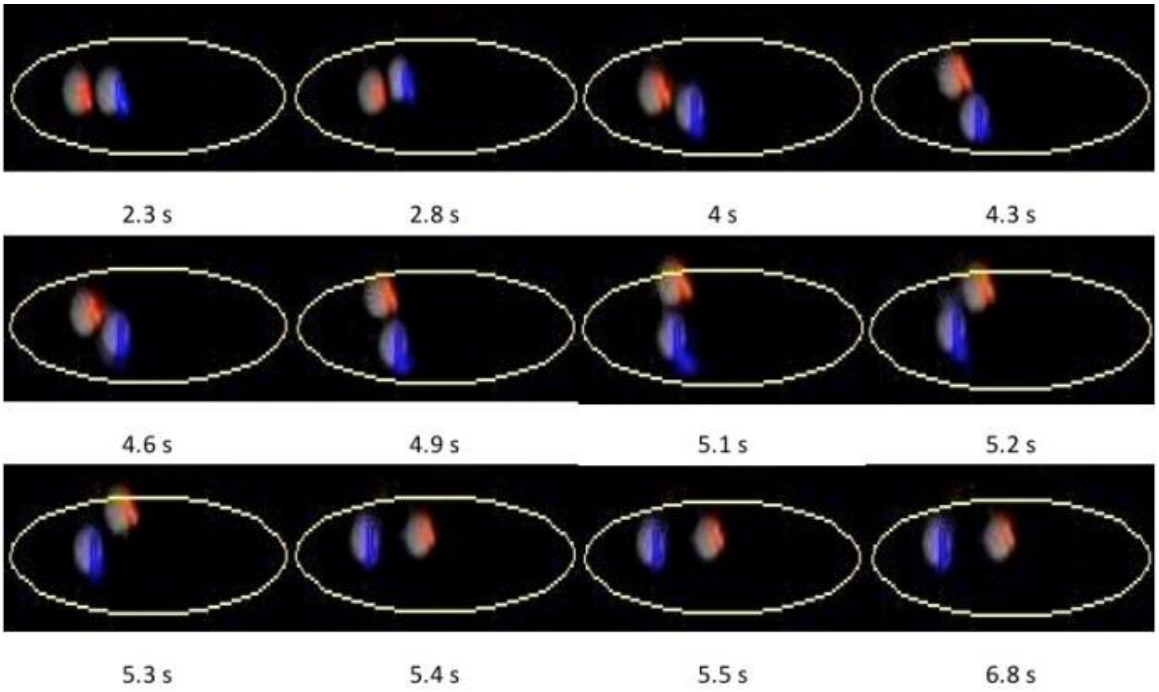

Figure 4: Zoom on SQR (red) bumping into α-HL under a 3 V/cm electric field in a buffer solution at pH = 7.2 with the time interval 2.3 s to 6.8 s extracted from video 1.

The striking feature is that, even though they have opposite charges, they do not stick to each other. This is a very interesting result, since objects with opposite charges could reasonably have been expected to attract each other. We suggest that they do not stick to each other because each object is surrounded by a belt of bound lipids allowing it to keep gliding along. Moreover, DDM molecules may still be present around the inserted trimeric SQR and could contribute to non-aggregation with the heptameric α-HL.

Figure 5 shows the behavior of the two objects at pH, 9.2, as a function of time. The complete sequence is shown in video 2. As figure 2 shows, the difference in charge is maximal at pH 9.2, meaning that this should be the pH at which separation is fastest and most efficient for this system.

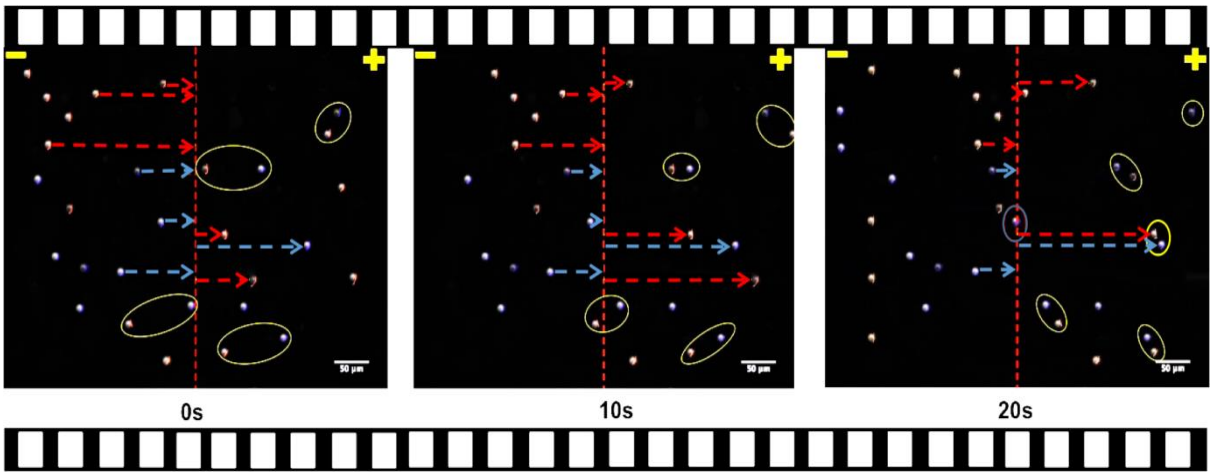

Figure 5: Images of fluorescent SQR and α-HL migration under a 3 V/cm electric field in a buffer solution at pH = 9.2 and recorded at 0, 10 and 20s extracted from video 2. The vertical red dotted line is an arbitrary reference line to highlight the position change of SQR and α-HL during the application of the electric field. Ellipses play the same role. The scale bar is 50 µm.

At this pH, all objects have a negative charge and race toward the positive electrode, at the right in the snapshot series, as shown by the red (for SQR) and blue (for α-HL) arrows.

SQR migrates faster (μ_{SQR} = 2.2x10^{-4} cm^2/V.s) than α-HL ($\mu_{\alpha\text{-HL}}$ = 6x10^{-5} cm^2/V.s), the mobilities being the same as when the proteins were alone. SQR either overtakes α-HL easily while migrating when they are in distinct lanes (as represented by the yellow ellipses) or may overtake by changing lanes when SQR catches up with α-HL on the same trajectory (see yellow ellipse on video 2 around t=13 s). Again, we observe no sticking to each other or overcrowding that would prevent separation.

At pH 6.8, the SQR remains immobile, while the α-HL moves toward the negative electrode (see video 3). At this pH, α-HL (blue spots) has a positive charge whereas SQR (red spots) has reached its *pI* (isoelectric point) [11, 13]. We measured the mobility of α-HL, and found µ = -1.86x10-5 cm^2/V.s, which is in good agreement with previous results [11].

The next experiment is intended to reverse roles for SQR and α-HL; previously, at pH = 8.3, α-HL reached its *pI* and SQR had a net negative charge. The SQR (in red) moves toward the positive electrode (see video 4) but the α-HL (in blue) is immobile. We measured the mobility of SQR, finding a value of 1x10^{-4} cm^2/V. s, which is in good agreement with our previous results [13].

All these scenarios at differing pHs will lead to the separation of the proteins, some of them more efficiently than others.

3.4 Separation of 3 objects (trimeric SQR + heptameric α-HL+ di-heptameric α-HL)

As a final experiment, we prepared a mixture of three objects: trimeric monotopic SQRs, heptameric α-HLs and dimers of heptameric α-HL. We constructed a system composed of a double bilayer into which we inserted these three objects, as described in the Materials and Methods section. The experiment was run at pH 9.2, where all objects are negatively charged and race each other toward the positive electrode. The complete sequence is shown in video 5. In this video, differing objects are shown in different shades of gray (trimeric SQR: the lightest, heptameric α-HL: medium gray and the dimer of heptameric α-HL: the darkest). Below, in figure 6, trimeric SQR and heptameric α-HL are shown in their usual colors, red and blue respectively, and the third object, the dimer, is in yellow (same color code for the arrows).

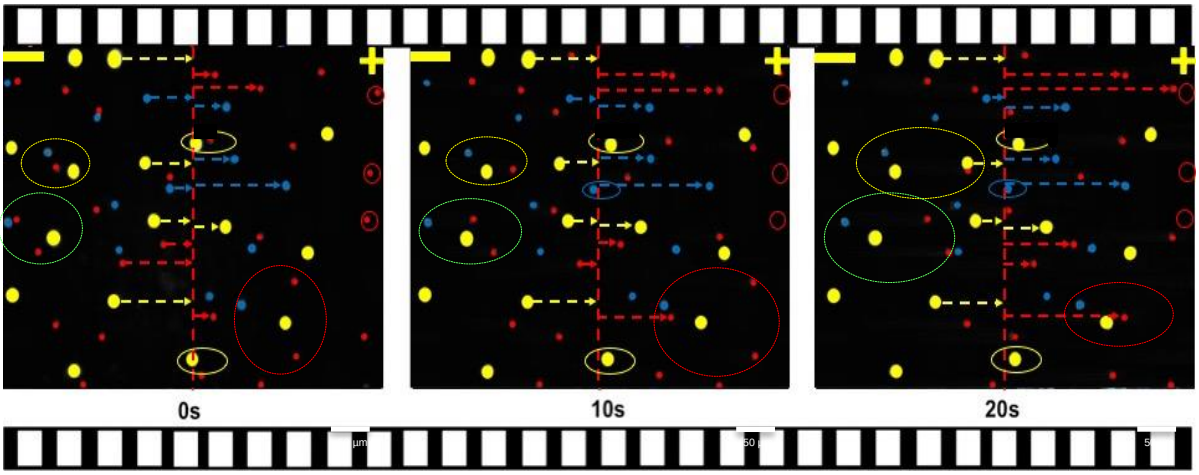

Figure 6: Images of fluorescent SQR, α-HL and dimeric α-Hl electrophoretic migration recorded at 0, 10 and 20s extracted from video 5. The vertical red dotted line is an arbitrary reference line to highlight the position change of objects. Dashed ellipses illustrate the relative evolution of typical situations where the 3 objects are visible. The scale bar is 50 µm.

Circles/ellipses illustrate situations where the three objects (red, blue and yellow) are close at the beginning and where distances between them increase with time. The fastest is SQR and the slowest is the dimer. Each object has its mobility value, determined during previous work on protein alone.

This final experiment shows that it is possible to electrophoretically separate a mixture of membrane proteins in supported lipid bilayers in a short time and with a fairly low electric field.

Mobility ($10^{-5}cm^2/V.s$) of	SQR			αHL			di- αHl	
System with *x* objects / pH	***x*= 1**	***x*=2**	***x*=3**	***x*= 1**	***x*=2**	***x*=3**	***x*=1**	***x*=3**
5.2-5.5	-20.6	-	-	-8.2	-	-	-	-
6.4	- 6.0	-	-	-	-	-	-	-
6.8	0	0	-		-1.9	-	-	-
7.2	1.6	1.6	-	-1.9	-2.1	-	- 1,0	-
8.3	9.9	10.0	-	0	0	-	-	-
9.2	22.0	22.2	21.8	5.8	6.0	6.1	-	1.9

Table 2: Mobilities of the different objects as a function of pH and of the mixture (±10%)

As found with 2 objects, we observe that the difference in mobilities is sufficient at pH 9.2, and induces an efficient separation.

All the mobilities recorded during these experiments are presented in table 2.

4. Conclusion

This work provides new insights into the feasibility of an electrophoretic separation of genuine membrane proteins in single, double and multi supported bilayers. It reveals that SQR can migrate alone while α-HL remains immobile or *vice versa*, which is achieved by suitably adjusting the pH of the buffer. We also demonstrated that when proteins are charged (mixture of 2 or 3 objects), separation is feasible due to the differing mobility that results from their differing structure and charges. If the proteins meet while migrating, they can escape each other and continue migrating, probably due to the existence of bound lipids which act as a bumper. Compared to standard electrophoretic methods, we observed clear separation of the three objects in substantially shorter distances (microns) and times (seconds), and with a weaker field (3V/cm). In addition, this system is easy to build using rehydration of dried lipidic film, and the resulting multibilayer should increase membrane protein loading capacities. This should be generalizable. The next challenge will be to insert unknown membrane proteins from living systems into the supported bilayer. We suggest two methods as potentially feasible: first, direct

fusion of cells on an already-present supported bilayer and second, repairing an intentionally-created scratch in a multibilayer with a low-detergent solubilized biological membrane before applying the electric field.

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Sample CRediT author statement

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Figure Captions

Figure 1: Schematic diagram of the cell used to carry out protein velocity measurements in double SLB: (a) video setup for electrophoretic mobility measurements: cell positioned on an inverted microscope equipped with a camera; (b) zoom on the system: SQR (red ellipses) and α-HL (blue mushrooms) inserted on top of double bilayer.

Figure 2: Electrophoretic mobility of trimeric SQR alone and heptameric α-HL alone as a function of pH (the error bar is ±10%).

Figure 3: Images of fluorescent SQR and α-HL migration under a 3 V/cm electric field in a buffer solution at pH = 7.2 and recorded at 0, 10 and 20s extracted from video 1. The scale bar is 50 µm. The vertical red dotted line is an arbitrary reference to highlight the position change of SQR and α-HL during the application of the electric field. Ellipses illustrate the motion of one object compared to the other. The scale bar is 50 µm.

Figure 4: Zoom on SQR (red) bumping into α-HL under a 3 V/cm electric field in a buffer solution at pH = 7.2 with the time interval 2.3 s to 6.8 s extracted from video 1.

Figure 5: Images of fluorescent SQR and α-HL migration under a 3 V/cm electric field in a buffer solution at pH = 9.2 and recorded at 0, 10 and 20s extracted from video 2. The vertical red dotted line is an arbitrary reference line to highlight the position change of SQR and α-HL during the application of the electric field. Ellipses play the same role. The scale bar is 50 µm.

Figure 6: Images of fluorescent SQR, α-HL and dimeric α-Hl electrophoretic migration recorded at 0, 10 and 20s extracted from video 5. The vertical red dotted line is an arbitrary reference line to highlight the position change of objects. Dashed ellipses illustrate the evolution of typical situations, especially where the 3 objects are visible. The scale bar is 50 µm.

Tables

Table 1: Systems, pH and incubation temperature in different supported lipidic bilayer systems

Mixture	Conditions	DMPC single bilayer	DMPC double bilayer	DMPC Multibilayer	EggPC Multibilayer
of 2 Objects	pH	6.8; 8.3; 9.2	-------	6.8; 8.3; 9.2	6.8; 7.2; 8.3; 9.2
	Incubation T(°C)	33	-------	33	33
of 3 Objects	pH	-------	9.2	-------	-------
	Incubation T(°C)	-------	33 °C (SQR & α-HL) then 22 °C to obtain α-HL dimers	-------	-------

Table 2: Mobilities of the different objects as a function of pH and of the mixture (±10%)

pH	**SQR x 10^{-5} (cm^2/V.s)**			**αHL x 10^{-5} (cm^2/V.s)**			**di- αHl x 10^{-5} (cm^2/V.s)**	
System	**1 Object**	**2 Objects**	**3 Objects**	**1 Object**	**2 Objects**	**3 Objects**	**1 Object**	**3 Objects**
5.2-5.5	-20.6	-	-	-8.2	-	-	-	-
6.4	- 6	-	-	-	-	-	-	-
6.8	0	0	-		-1.9	-	-	-

7.2	1.6	1.6	-	-1.9	-2.1	-	- 1,0	-
8.3	9.9	10	-	0	0	-	-	-
9.2	22	22.2	21.8	5.8	6	6.1	-	1.9